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Transcription of Yeast Mitochondrial DNA by *E. coli* RNA Polymerase

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Yeast mitochondrial DNA was transcribed by *E. coli* RNA polymerase with or without sigma, and the starting nucleotide sequences of synthesized RNA were analyzed. RNA polymerase holoenzyme predominantly initiated RNA synthesis with the sequence of pppAU—, whereas the enzyme lacking sigma permitted random initiations. The sigma factor appears to restrict initiation to a limited number of sites even in a heterologous system.

INTRODUCTION

Recent experiments have established in bacterial systems that a protein factor named "sigma" is responsible for specific initiation of transcription at promoters of chromosomal and bacteriophage DNA's.[1~4] Thereafter many attempts have been made to elucidate the function of the sigma factor. However, the molecular mechanism by which sigma acts is still obscure. One of approaches to this question is to examine the function of this factor with DNA from heterologous systems in which the promoters might have different structures from those of *E. coli* DNA. Several investigations on this subject have been done by using DNA of mammalian chromatin,[5,6] animal virus[7~9] and mitochondria,[10~12] and conflicting results have been obtained. In the present study, RNA transcribed on yeast mitochondrial DNA by *E. coli* RNA polymerase was characterized by using the technique labelling the starting termini of RNA. It was found that the sigma factor played a function restricting initiation to a limited number of sites even in this heterologous system.

EXPERIMENTAL

Preparation of yeast mitochondrial DNA: *S. cerevisiae* strain D206 (wild type) was obtained from Dr. Katsume, grown at 28°C with constant shaking in the medium of Ohnishi *et al.*,[13] and harvested at a mid-log phase. Mitochondrial DNA was prepared from the isolated mitochondrial fraction by the following two alternative procedures.

Method A: Yeast spheroplasts were prepared with glusulase as described by Duell *et al.*[14] and lysed using a mechanical homogenizer. Liberated mitochondria were treated for 20 min at 0°C with 50 μg per ml DNase-1[15] in 20% sucrose-10 mM $MgCl_2$-1 mM EDTA in 5 mM potassium phosphate buffer (pH 6.8) and washed three times with 0.25 M sucrose-0.1 M EDTA (pH 8.0), and lysed in 15 mM NaCl-1.5 mM sodium citrate con-

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taining 1% SLS (sodium lauryl sulfate) by heating at 60°C for 10 min. Pronase was added to a final concentration of 100 μg per ml. The mixture was incubated for 2 hr at 37°C and then frozen overnight. After thawing, the mixture was treated with an equal volume of phenol equilibrated with 1% SLS-20 mM Tris-buffer (pH 9.0). The separated aqueous fraction was treated with pancreatic RNase for 30 min at 37°C to digest any contaminating RNA. After removing RNase by the phenol treatment, the solution was dialyzed against 0.1 mM EDTA-5 mM potassium phosphate buffer (pH 6.8) and then passed through a Sepharose 2B column equilibrated with the same buffer used for dialysis.

Method B: Treatment of the mitochondrial preparation with DNase in Method A was omitted. Instead, CsCl was added to the final DNA fraction to 1.690 g/ml and the solution was centrifuged for 48 hr at 37,000 rev/min. The mitochondrial DNA fraction was collected and dialyzed against 0.1 mM EDTA-potassium phosphate buffer (pH 6.8).

The DNA preparation gave a single band with a density of 1.683 g/ml on CsCl density-gradient centrifugation, indicating no contamination of nuclear DNA (Fig. 1). On sucrose density-gradient centrifugation, both preparations showed a broad distribu-

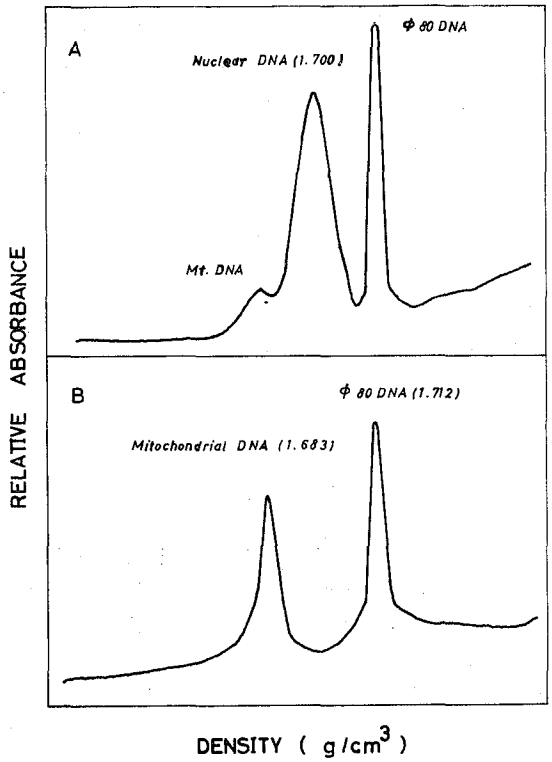

Fig. 1. Density tracing of analytical CsCl-density gradients of DNA. (A) DNA from whole cells (B) mitochondrial DNA.

DNA was dissolved in 1.690 g/ml of CsCl and centrifuged for 22 hr at 44,770 rev/min and 25°C in the Spinco model E analytical ultracentrifuge. The peak on the right in each tracing corresponds to ϕ80 DNA as a density marker (1.712).

tion, ranging from about 16 s to 40 s. Electron microscopy of the DNA preparation showed linear molecules with the size distribution from 1 μ to 9 μ.

Purification of *E. coli* RNA polymerase: This was prepared from *E. coli* A19 as described in reference.[16] The purification procedure involved the following steps: cell disruption, high speed centrifugation, streptomycin treatment, ammonium sulfate fractionation (35% to 45% saturation), DEAE-cellulose column chromatography (0.13 M to 0.25 M KCl fraction) and two cycles of Agarose (BioRad A-5 m) column chromatography at high and low salts. The final enzyme preparation was concentrated by dialyzing against 10 mM $MgCl_2$-50 mM KCl-0.1 mM dithiothreitol-50 mM Tris (pH 7.8)-50% glycerol, and stored at −20°C. The enzyme was fractionated into the core enzyme and sigma by phosphocellulose (Whatman P11) column chromatography according to the method of Burgess *et al.*[2]

Conditions used for RNA synthesis: Synthesis of RNA was carried out in the reaction mixture containing 8 mM $MgCl_2$, 80 mM KCl, 40 mM Tris (pH 7.8), 0.4 mM (^{3}H)-ATP (3000 cpm/mμmole), 0.4 mM each of CTP, GTP and UTP, 30 μg/ml of holoenzyme or 15 μg core enzyme, and 5 μg/ml of DNA as template. Incubation was usually carried out for 20 min at 37°C. For assay of the extent of RNA synthesis, reaction was terminated by adding cold 10% TCA. The precipitate formed was collected on a glass filter (Whatman GF/C), dried and the radioactivity was determined. For labelling the starting termini of RNA, ATP or GTP in the above reaction mixture was replaced by 0.1 mM of (γ-^{32}P)-ATP or (γ-^{32}P)-GTP. After incubation, the reaction mixture was treated with 80% phenol. The separated aqueous layer was passed through a Sephadex G 100 column (1 cm × 20 cm) to remove substrates. The RNA fractions were pooled. Nucleoside (γ-^{32}P)-triphosphates with a specific activity of about 2–5 × 10^9 cpm/μmole were prepared by the method of Glynn and Chappell.[17]

Measurement of ribonuclease resistance: The RNA synthesizing mixture containing (^{3}H)-ATP was prepared as in the above section. Following incubation for 20 min at 37°C, 10 μg/ml of pancreatic DNA was added, and incubation was continued for an additional 5 min. The RNA fraction was isolated by using a Sephadex G 100 column. The labelled (^{3}H)-RNA thus prepared was incubated at 66°C for 20 hr in 0.15 M NaCl-0.015 M sodium citrate for self-annealing. Heat-denatured RNA was prepared by heating to 100°C for 10 min and chilling. To test RNase resistance, the RNA solution (0.5 μg/ml) was incubated with 2 μg pancreatic RNase and 0.10 units RNase T1 at 25°C.[18] 0.1 ml aliquots were withdrawn at different times, precipitated with trichloroacetic acid, and counted the radioactivity.

Separation of oligonucleotides: Oligonucleotides produced from RNA by pancreatic and T1 RNases were separated by chromatography on DEAE-Sephadex (A 25) columns according to the method of Takanami.[19] The separation of dinucleotides with different sequences was carried out by chromatography on Dowex-1 (X2) columns.

RESULTS

Template activity of mitochondrial DNA: Yeast mitochondrial DNA was transcribed efficiently by *E. coli* RNA polymerase as shown in Fig. 2. The rate of synthesis was higher than those of calf thymus DNA and poly d(AT). Transcription by

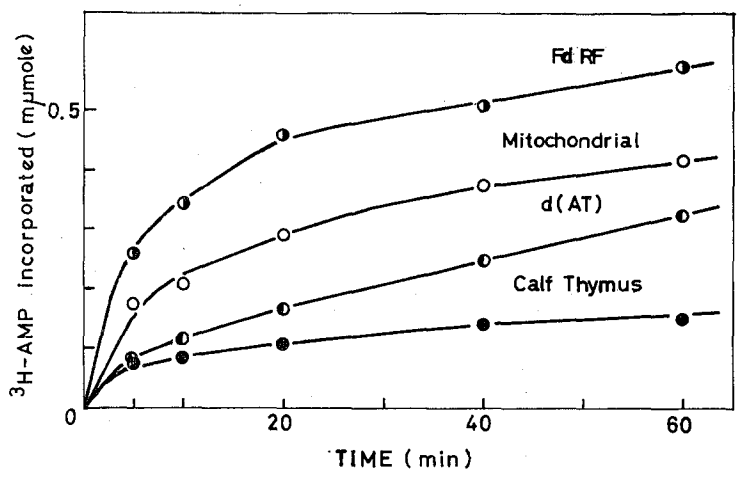

Fig. 2. Time course of RNA synthesis on different templates.
The composition of reaction mixtures (1 ml each) was given in the text. 0.1 ml each was withdrawn at the time indicated and the incorporation of (^{3}H)-AMP into RNA was determined.

RNA polymerase core enzyme was stimulated two or three fold by the addition of the sigma factor. The number of initiation sites for *E. coli* RNA polymerase holoenzyme on mitochondrial DNA was estimated by the method of Bautz *et al.*[20] As described in the next section, RNA synthesis by holoenzyme was predominantly initiated with ATP. Accordingly, the DNA-enzyme complex was first formed in the absence of substrates and then incorporation of (γ-^{32}P)-ATP was measured in the presence of rifampicin and substrates. In Fig. 3, the saturation curve for mitochondrial DNA is compared with that for bacteriophage fd replicative form (RF) DNA containing one ATP-initiation site per genome.[21] Under the similar conditions, the plateau values obtained for fd RF DNA and mitochondrial DNA corresponded to about 1.2 and 11.7 initiation sites per 10^4 nucleotide pairs, respectively. Assuming that the molecular length of intact yeast mitochondrial DNA is about 25 μ,[22] the number of initiation sites per mitochondrial DNA is more than 30. This value appears to be very high compared with other determinations.[20] The degree of symmetry in synthesized products was analyzed by means of a self-annealing method. About 30% of the self-annealed RNA was resistant to RNases, whereas heat denatured RNA was almost completely degraded. This result suggests that some regions of DNA were transcribed in the both directions.

Starting nucleotide sequences of RNA synthesized with and without sigma: It has been reported that RNA chains formed by both *E. coli* RNA polymerase holoenzyme and its core enzyme are predominantly initiated with purine nucleoside triphosphates.[4,23] Accordingly, the relative incorporation of ^{32}P from each of (γ-^{32}P)ATP and (γ-^{32}P)-GTP into RNA synthesized on mitochondrial DNA by *E. coli* RNA polymerase was investigated. The results are shown in Table I. ATP was predominantly used for chain initiation by the holoenzyme, whereas both ATP and GTP were incorporated by the core enzyme. To investigate the subsequent nucleotide sequences, RNA labelled with either (γ-^{32}P)-ATP or (γ-^{32}P)-GTP was prepared in the presence and absence of the sigma factor. The labelled RNA was hydrolyzed with pancreatic RNase and resulting fragments were

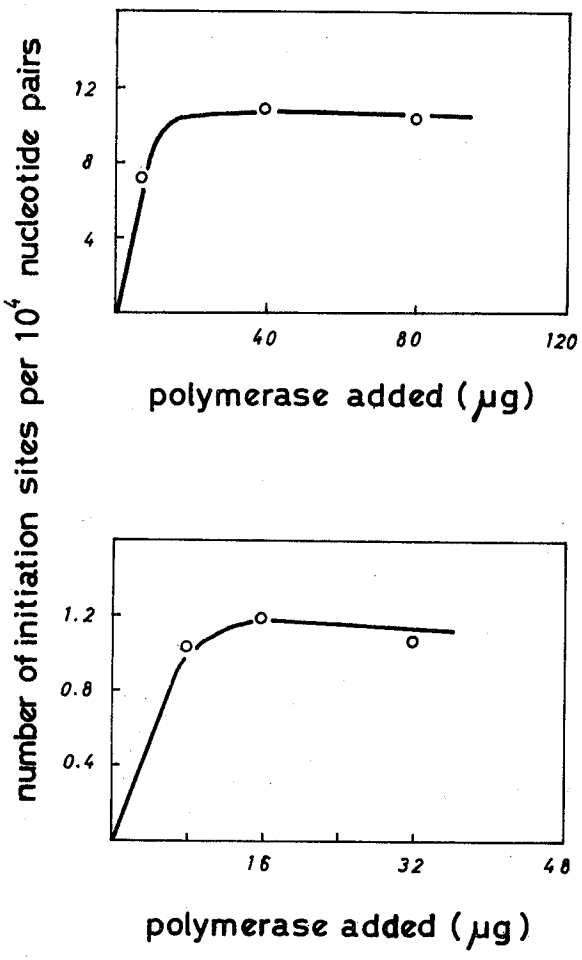

Fig. 3. Number of initiation sites with ATP on mitochondrial DNA (A) and fd RF DNA (B).

Indicated amounts of *E. coli* RNA polymerase were incubated at 17°C in a reaction mixture (1 ml): 40 μmoles Tris-HCl (pH 7.9), 8 μmoles $MgCl_2$, 80 μmoles KCl and 2.5 μg of either mitochondrial DNA or fd RF DNA. After 20 min, 0.1 μmole (γ-^{32}P)-ATP (specific activity, 2–5×10^9 cpm/μmole) and 0.4 μmole each of other three nucleotide triphosphates were added together with 10 μg of rifampicin. The mixture was incubated at 37°C for 20 min. Synthesized RNA was isolated as described in the text and the incorporation of (γ-^{32}P)-ATP was determined.

separated by chromatography on DEAE-Sephadex columns. The results are shown in Table II (a) and (b). The chromatographic pattern of pancreatic RNase hydrolysate of RNA labelled with (γ-^{32}P)-ATP is shown in Fig. 4. With RNA formed by RNA polymerase holoenzyme, about 80% of ^{32}P radioactivity appeared at the pppApPyp region. In order to identify the species of the second nucleotide, the pppApPyp fraction was collected. Two ribonucleoside diphosphates and four ribonucleoside triphosphates were added as markers and the mixture was chromatographed on a Dowex-1 column. About 90% of ^{32}P was found at the pppApUp region (Fig. 5). On the contrary, the starting

Table I. Incorporation of Purine Nucleoside (γ-^{32}P)-triphosphates into RNA.

Enzyme	Nucleoside (γ-^{32}P)-triphosphates incorporated (pmoles)		
	ATP	GTP	ATP/GTP
Holo enzyme	14.40	4.20	3.43
Core enzyme	2.41	2.44	0.99

Reaction mixtures containing either (γ-^{32}P)-ATP or (γ-^{32}P)-GTP were prepared and incubated for 20 min at 37°C. The radioactivity in the RNA fractions was determined as described in the text.

Table II. Recovery of ^{32}P in the Fragments Produced from RNA Labelled with (γ-^{32}P)-ATP and (γ-^{32}P)-GTP by Pancreatic RNase Digestion.

A. Holo enzyme

Fragments	Recovery %	Fragments	Recovery %
pppApPyp	60.4	pppGpPyp	10.9
pppApPupPyp	11.6	pppGpPupPyp	5.2
pppAp(Pup)$_2$Pyp	1.8	pppGp(Pup)$_2$Pyp	2.9
pppAp(Pup)$_3$Pyp	1.4	pppGp(Pup)$_3$Pyp	1.2
≥pppAp(Pup)$_4$Pyp	2.1	≥pppGp(Pup)$_4$Pyp	2.5

B. Core enzyme

Fragments	Recovery %	Fragments	Recovery %
pppApPyp	31.3	pppGpPyp	20.1
pppApPupPyp	6.3	pppGpPupPyp	10.7
pppAp(Pup)$_2$Pyp	6.9	pppGp(Pup)$_2$Pyp	9.9
pppAp(Pup)$_3$Pyp	1.3	pppGp(Pup)$_3$Pyp	4.8
≥pppAp(Pup)$_4$Pyp	4.0	≥pppGp(Pup)$_4$Pyp	4.7

RNA labelled with (γ-^{32}P)-ATP and (γ-^{32}P)-GTP were synthesized with or without the sigma factor and prepared as in the text. The labelled RNA were hydrolyzed with pancreatic RNase and the resulting radioactive fragments were separated by DEAE-Sephadex column chromatography as described in the lenged to Fig. 4.

sequences of RNA synthesized by the core enzyme randomly distributed. These results clearly indicate that the sigma factor restricts the initiation of RNA synthesis to a unique sequence of pppAU—. In order to analyze the sequences subsequent to pppAU—, the T1 RNase hydrolysate of (γ-^{32}P)-ATP labelled RNA was chromatographed on a DEAE-Sephadex column with the conditions used for analysis of the pancreatic RNase hydrolysate. However, a heterogeneous pattern was obtained, indicating that RNA chains initiated with pppAU— were different in the subsequent nucleotide sequences.

DISCUSSION

The specificity of RNA chain initiation was investigated by analyzing the starting nucleotide sequences of RNA synthesized. This method provides more sensitive criterion

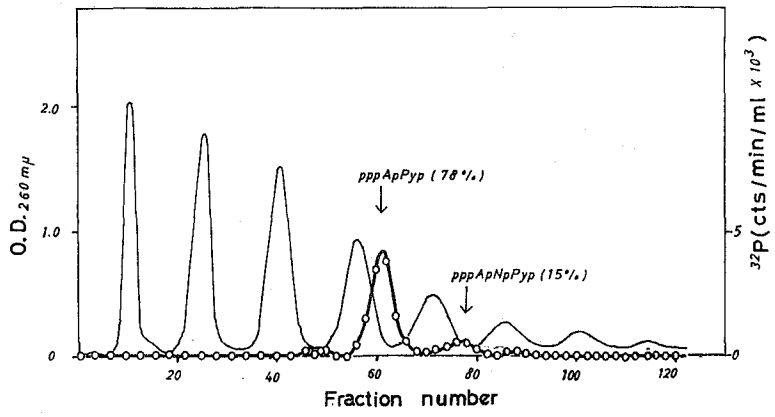

Fig. 4. A typical chromatographic pattern of the pancreatic RNase hydrolysate of RNA labelled with (γ-^{32}P)-ATP.

RNA labelled with (γ-^{32}P)-ATP was prepared and non-radioactive RNA was added to provide markers. The mixture was hydrolysed with pancreatic RNase and the hydrolysate was chromatographed on a DEAE-Sephadex (A25) column (0.5 cm×20 cm) with a convex gradient from 0.08 M NaCl-7 M urea to 0.4 M NaCl-7 M urea (pH 8.) After chromatography, the O.D.$_{260}$ and ^{32}P radioactivity were determined. Arrows show the positions of possible fragments containing the end.

—○—○—: ^{32}P radioactivity ————: optical density at 260 mμ

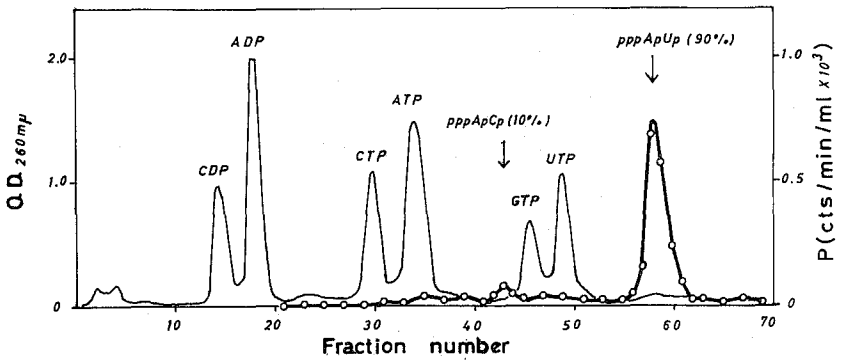

Fig. 5. Dowex-1 column chromatography of the pppApPyp fraction produced from RNA labelled with (γ-^{32}P)-ATP.

The pppApPyp fraction isolated by DEAE-Sephadex column chromatography from the pancreatic RNase hydrolysate was diluted fivefolds. Markers were added and the mixture was chromatographed on a Dowex-1 (X2)-400 mesh column with a linear gradient from 0.01 N HCl to 1.0 M NaCl-0.01N HCl. After chromatography, the O.D.$_{260}$ and ^{32}P radioactivity were determined.

—○—○—: ^{32}P radioactivity ————: optical density at 260 mμ

than others, as it can distinguish the specific transcription from the non-specific one precisely. The results shown in Figs. 4 and 5 clearly demonstrated that RNA with unique starting sequence was predominantly synthesized in the presence of sigma. By contrast, RNA polymerase lacking this factor appears to initiate RNA synthesis with many different sequences. The result is very similar to those obtained in a homologous system,

consisting of phage DNA as template and *E. coli* RNA polymerase.[4]

It is known that mitochondria contains its own transcription system.[24] RNA polymerase which specifically required mitochondrial DNA as template has been isolated from *Neurospora*.[25] The molecular structure of this enzyme is distinct from those of nucleolar and nucleoplasmic enzymes and from the bacterial enzyme employed in this report. Therefore, it is reasonable to assume that the structure of promoters in mitochondrial DNA is different from those recognized by enzymes of heterologous systems. Nevertheless, it was shown in the transcription of mitochondrial DNA by *E. coli* RNA polymerase that the sigma factor functioned just like in the bacterial system. Several explanations would be possible for this phenomenon. There is a possibility that the promoters in mitochondrial DNA have the structure similar to that of promoters in the bacterial system, even though each system has different enzyme. This being the case, bacterial enzyme might initiate RNA synthesis at the real promoter sites of mitochondrial DNA. The other interpretation of the result would be that mitochondrial DNA contains the structure similar to that of *E. coli* promoters, although the sites of such the structure are different from those at which mitochondrial enzyme initiates RNA synthesis. *E. coli* enzyme might initiate RNA chains at such sites. The latter interpretation is more likely, because of that mitochondrial DNA contains about 80% A : T and that the promoters of phage DNA have a high AT content.[26] Estimation of initiation sites per genome also gave a high value (Fig. 3). Validity of these interpretations would be judged by comparing the initiating sequences with those of RNA formed by mitochondrial enzyme.

In accordance with the observations on the bacteriophage DNA-directed system, the sigma factor apparently restricts initiation of RNA synthesis to specific sites in the heterologous system. It is known that *E. coli* RNA polymerase core enzyme has an intrinsic property to initiate RNA randomly on many DNA templates including synthetic polymers.[4] The sigma factor itself has no affinity to DNA. It is likely that the sigma factor prevents random initiation of RNA synthesis by associating with the core enzyme.

Intact mitochondrial DNA is shown to have a circular form with a contour length of 25 μ.[22] As shown by electron microscopy, DNA used in the present study was linear and heterogeneous in size, whereas a great care was taken to avoid mechanical shaking. This indicates that the intact molecule was broken into fragments during the preparation. Nevertheless, RNA formed by RNA polymerase holoenzyme had a unique starting sequence. It is shown that RNA polymerase has strong affinity to single stranded regions of template. When DNA fragments formed by DNase-treatment was used as template, RNA synthesis was mostly initiated at the terminal region.[26] It is therefore suggested that the template used in the present study did not contain such single stranded regions at the termini. Analysis of the terminal nucleotides also suggested that breakage of the DNA molecule did not occur randomly, but at specific sites (unpublished observations). It is of interest to speculate that a specific endonuclease which produces duplex cleavages on double-stranded DNA might exist in mitochondria.

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